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14. ABSTRACT
A variety of unique metal mixtures have entered the military arsenals of many countries in recent years. One such material is the tungsten alloys, which have been proposed as replacements for depleted uranium (DU) in armor-penetrating munitions. As a result, opportunities for exposure are increasingly likely. This leads to questions, similar to those originally surrounding DU, as to the health effects of exposure to the tungsten alloys, especially for embedded fragment exposures. The Armed Forces Radiobiology Research Institute (AFRRI) recently performed research that showed one of the militarily promising tungsten alloys to be a potent carcinogen when implanted in rats. The need to confirm the carcinogenicity of such alloys in another rodent species is an important second step required in biological as well as regulatory terms to better assess the cancer risk in humans. Results of this work will help in formulating policies for military surgeons who must treat personnel wounded by fragments of the alloys. Indications of unacceptable risks of exposure will also help determine the advisability of deploying (or developing) similar munitions. In year 4 of this project, metal-implanted mice in the two-year carcinogenicity study continue to be assessed for tumor development. At present, no tumors have been observed in the tungsten alloy implanted mice, but tumors have begun to form around the nickel pellet (positive control) after approximately 60 weeks post-implantation.

15. SUBJECT TERMS
Tungsten alloy; carcinogenicity; munitions; mice

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INTRODUCTION

A variety of unique metal mixtures have entered the military arsenals of many countries in recent years. One such material is the tungsten alloys, which have been proposed as replacements for depleted uranium (DU) in armor-penetrating munitions. As a result, opportunities for exposure are increasingly likely. This leads to questions, similar to those originally surrounding DU, as to the health effects of exposure to the tungsten alloys, especially for embedded fragment exposures. The Armed Forces Radiobiology Research Institute (AFRRI) recently performed research that showed one of the militarily promising tungsten alloys to be a potent carcinogen when implanted in rats. The need to confirm the carcinogenicity of such alloys in another rodent species is an important second step required in biological as well as regulatory terms to better assess the cancer risk in humans. Results of this work will help in formulating policies for military surgeons who must treat personnel wounded by fragments of the alloys. Indications of unacceptable risks of exposure will also help determine the advisability of deploying (or developing) similar munitions. The National Toxicology Program (NTP) Two-Year Study Protocol carried out in two rodent species is the recommended approach in the U.S. for identifying human carcinogens. This investigation aims to confirm the previous AFRRI data in rats by carrying out a two-year protocol in mice based upon NTP guidelines. The study uses the B6C3F1 hybrid mouse, a strain commonly used in carcinogenicity and toxicity assessment studies, implanted with pellets of tungsten alloys, the individual component metals of the alloys, tantalum (negative control), or nickel (positive control). The protocol includes serial collection of tissues 1, 3, 6, and 12 months post-implantation aimed at identifying early changes relevant to the development of carcinogenic endpoints.

BODY

AFRRI research recently showed that mixtures of tungsten, nickel, and cobalt are tumorigenic and genotoxic in HOS cells (1) and that embedded pellets of the alloy tungsten-nickel-cobalt cause cancer in rats (2

). However, studies with cultured cells and rats are not in themselves sufficient to allow designation of a substance as carcinogenic in humans. In general, the National Toxicology Program (NTP) and the Environmental Protection Agency (EPA), two agencies involved in cancer risk determination, agree that convincing evidence that the agent is probably carcinogenic in humans is obtained if the agent demonstrates carcinogenic potential in two rodent species, using the NTP two-year carcinogenicity protocol. This study proposes to obtain that data.

We hypothesize that the alloys tungsten/nickel/cobalt and tungsten/nickel/iron are carcinogenic in the mouse as indicated by the NTP two-year carcinogenicity protocol. Our test of this hypothesis incorporates the following Aims.

Aim 1: Determine whether the alloys tungsten-nickel-cobalt and tungsten-nickel-iron cause cancer in mice. Include in the protocol mice embedded with pellets of the individual metals composing the alloys and the various metal combinations (blended with biologically inert tantalum at the same percentages present in the alloys).

Pellets of the alloys or the various component metals will be implanted in the leg muscles of mice, and mice will be maintained and monitored for 24 months post-implantation. The response in alloy-implanted mice will be compared to mice implanted with pellets of 100% nickel, a known carcinogen (positive controls) and mice implanted with tantalum, an inert metal used in prosthetic devices (negative control). At the end of the study or whenever sacrifice of participating mice is required, necropsies will be performed to obtain evidence of tumor development. Data of tumor sites and incidence will be compiled. Tumors will be histologically examined and classified.

Aim 2: Sacrifice mice at various times after alloy implantation to detect early signs of tumor development.

Subgroups of animals treated identically to the mice described in Aim 1 will be euthanized 1, 3, 6, and 12 months after metal implantation to identify any early signs of histopathology associated with exposure to the implanted metals.

Aim 3: Measure tissue levels of the various metals that compose the alloys to correlate metals levels with tumor development.

Levels of W, Ni, Fe, Co, and Ta will be measured in organs from the animals used in Aims 1 and 2. Data will be used to relate tissue metal levels to any cancer incidence observed in those particular tissues. These data will allow a correlation of tissue metal content with tumor development.

Methods and Experimental Design

The B6C3F1 mouse will be used for these experiments. The hybrid B6C3F1 mouse is commonly used in a wide variety of research applications, particularly toxicology, and is the strain recommended by the National Toxicology Project for two-year carcinogenicity investigations. A study employing 1200 mice will provide a sufficient number to perform the described two-year carcinogenicity assessment protocol using fifteen treatment groups, serial sacrifices to test for early changes in exposed animals, and sufficient additional animals to serve as colony sentinels and backups.

The project focuses on two tungsten alloys of special interest to the military: 91.1% tungsten/6% nickel/2.9% cobalt and 91% tungsten/7% nickel/2% iron. All of the tests include fifteen treatment groups consisting of various controls, tungsten alloy metal tests, and a toxicity reference metal (lead). Exposures are accomplished by implantation of the metals as pellets in the form of cylinders 1 mm in diameter and 2 mm long. Alloy pellets are custom-fabricated using sintered metal powder technology similar to that

used for military munitions. Pellets designed to test individual metals in the alloys contain the same percentage content by weight as the full alloy, with the balance made up with the biologically inert metal tantalum. Lead and nickel pellets (toxicity reference metal and positive control, respectively) are cut from wires of pure metal and formed to a dimension identical to the alloy pellets. The individual test groups are described as follows:

1. Sham-implantation controls
2. Tantalum pellet-implanted (implantation controls)
3. Nickel (100%) pellet-implanted (positive controls)
4. Lead (100%) pellet-implanted (reference metal)
5. Tungsten/nickel/cobalt pellet-implanted
6. Tungsten/nickel/iron pellet-implanted
7. Tungsten/tantalum pellet-implanted
8. Nickel/tantalum pellet-implanted
9. Cobalt/tantalum pellet-implanted
10. Iron/tantalum pellet-implanted
11. Tungsten/nickel/tantalum pellet-implanted
12. Tungsten/cobalt/tantalum pellet-implanted
13. Tungsten/iron/tantalum pellet-implanted
14. Cobalt/nickel/tantalum pellet-implanted
15. Iron/nickel/tantalum pellet-implanted

Two-Year Carcinogenicity Study (Aim 1)

These experiments test the carcinogenic potential of two doses of pellets implanted in mice for 24 months. Twenty male mice are used in each treatment group. The doses used and manner in which the animals are exposed are based on successful mouse and rat pellet implantation models designed at AFRRI and used for nearly a decade. Mice are weighed on a weekly basis and observed for any changes indicative of developing pathology. At the end of the 24-month period or at any time mice appear moribund, they will be sacrificed. At the time of sacrifice, blood will be drawn for a complete hematological and clinical assessment, and the mice will undergo full

necropsy, preserving selected tissues and organs and preparing slides for histopathological examination as required.

Progress to Date: All mice in the 24 month experimental groups have been implanted. We are now 15 to 18 months (depending on the experimental group) into the 24 month period and no pellet-associated tumor formation has been observed with the exception of the Ni-implanted group which serves as our positive control. However, survival of the high-dose (4 pellet) Ni group averaged 60 weeks before requiring euthanasia. In contrast, the high-dose Ni-implanted rats required euthanasia by 24 weeks after treatment. This species difference is also seen with respect to the W/Ni/Co alloy. Tumor development in the F344 rat was apparent by 14 weeks, yet no tumor development was observed in the B6C3F1 mouse after 56 weeks. This lengthy latency period is not totally unexpected. Reports in the literature indicate that the B6C3F1 mouse is more refractory to tumor development, especially in the skeletal muscle system, compared to other mouse strains such as the Balb/C and C3H (3,4).

As noted above, all mice are weighed weekly during our health status examination (palpitation of pellet implantation sites, overall assessment of well-being). Body weight has been shown to be an excellent indicator of overall health in rodents, as well as a sign of systemic toxicity as a result of experimental treatments. Figures 1-8 (Appendices) shows body weight gain of both low- and high-dose groups. The figures are separated into 4 groups for each dose: (1) control (sham, Ta) and alloys (WNiCo, WNiFe); (2) controls, positive control (Ni), and reference metal (Pb); (3) controls and single test metal compositions (WTa, NiTa, CoTa, FeTa); and (4) controls and double test metal compositions (WNiTa, WCoTa, WFeTa, NiFeTa, NiCoTa). With several exceptions, most mixtures did not affect weight gain. Nickel-implanted groups, both low- and high-dose, exhibited decreased body weight gain compared to controls. In addition, mice implanted with the single test metal compositions (NiTa, CoTa, FeTa) also gained weight at a slower rate than controls, but only in the high-dose group.

Year 4 Milestones: The scheduled euthanasia of both the low- and high-dose sets of the 24 month experimental groups will occur in Year 4. As noted above, a complete necropsy examination, as well as tissue collection for histopathology and metal analysis, will be conducted for each animal.

Serial Sacrifice Study (Aim 2)

The serial sacrifice study runs in parallel with the two-year carcinogenicity study and also includes the fifteen treatments groups. Ten male mice are employed in each treatment group. One, 3, 6, and 12 months after pellet implantation, mice are sacrificed, and gross pathologies performed. Selected tissues are collected and preserved and hematological tests performed. Histopathological surveys of selected animals will determine whether more extensive histopathology will be performed.

Progress to Date: In Year 3, the 12-month experimental groups have been implanted and have been followed as described above. No adverse health effects have been observed in these groups. In addition, all mice in the 1-month experimental group have been implanted, reached their experimental endpoint, and have been euthanized. There were no significant histopathology findings from any of the 1-month experimental groups and no differences were observed in weight gain between the experimental groups (Appendices, Fig. 9). However, there were significant differences in organ/body weight ratios for several of the experimental groups. In particular, spleen/body weight and liver/body weight ratios for the Ni, WNiCo, NiTa, CoTa, FeTa, and WNiTa groups were significantly higher than control (Appendices, Figs. 10 and 12). Conversely, kidney/body weight ratios were significantly lower in the 1-month experimental groups implanted with WNiCo, WNiFe, WNiTa, and WCoTa (Appendices, Fig. 11). Testes/body weight ratios were not affected by any of the experimental treatments in the 1-month groups.

Year 4 Milestones: In Year 4, the 3- and 6-month groups will be implanted and euthanized. The 12-month group will also reach its scheduled endpoint. As described above, a complete necropsy will be performed and relevant tissue samples collected for histopathology and metal measurements.

Metal Levels in Tissue (Aim 3)

Metal levels in tissues obtained from mice in the two-year carcinogenicity (Aim 1) and serial euthanasia (Aim 2) studies are being analyzed for metal content using inductively coupled-plasma mass spectrometry (ICP-MS). In addition, blood, serum, and

urine samples are also being assessed for metal content. The following metals are being measured: W, Ni, Co, Fe, Pb, and Ta.

Progress to Date: In Year 3, sample processing procedures have been streamlined to minimize the use of expensive ultrapure nitric acid. In addition, our ICP-MS instrument has undergone an extensive overhaul and upgrade. We wanted to complete the instrument upgrade before embarking on sample analysis in order to prevent lengthy disruptions in the middle of the sample analysis schedule. Samples (both tissue and body fluids) from the 1-month implantation group are currently be analyzed.

Year 4 Milestones: As complete sets of tissue and fluid samples become available, they will continue to be processed and analyzed.

KEY RESEARCH ACCOMPLISHMENTS

- All low- and high-dose 24-month mice have been implanted and are being followed to assess health effects of the implanted metals.
- Mice in the 24-month high-dose Ni group have begun to develop tumors at the pellet implantation site. No tumors have formed as yet in any other experimental group.
- Mice in the 12-month group of the serial euthanasia section have been implanted.
- All mice in the 1-month serial euthanasia section have been implanted, reached their experimental endpoints, and have been euthanized. The mice showed no adverse effects of metal implantation, although some perturbations in organ/body weight ratios and hematological parameters were observed.

REPORTABLE OUTCOMES

Oral Presentations

Kalinich, JF (14 March 08) Health Effects of Embedded Fragments. AFRRI Seminar. Bethesda, MD.

Kalinich, JF (12 August 08) Carcinogenicity of Embedded Tungsten Alloys. Force Health Protection Conference. Albuquerque, NM.

CONCLUSION

In Year 3, significant progress was made on this project. We continue to follow the mice in the 24-month implantation groups and currently only the mice in the high-dose nickel (positive control) group have developed tumors at the pellet implantation site. Although tumor development was much later than that observed in the F344 rat (2), it was not unexpected considering the long latency period for implanted-metal carcinogenesis in the B6CeF1 mouse reported by others (3,4). Therefore, if the tungsten alloys prove carcinogenic in this study, we would expect to see tumor development within the next 3-4 months. The 12-month groups of the serial section arm of the project have also been implanted and exhibit no signals of adverse health effects thus far. Also in Year 3, the 1-month groups were implanted and reached their euthanasia endpoint. Although no overt health effects were observed, there were significant effects of some of the treatments on both hematological parameters as well as organ/body weight ratios. It will be interesting to see whether these effects are only transient in nature when the 3, 6, and 12 month groups are completed.

Because of the large number of mice that must be examined and weighed weekly, we moved the implantation and euthanasia of the 3- and 6-month groups to Year 4. As detailed in the Year 2 Annual Report, the turmoil created by the expected retirement of the original Principal Investigator of this project, as well as his budgetary miscalculations at the start of the project, has resulted in far less technical help than required. As a result, this has led to extensive modifications in the surgical implantation schedule, so that we might

obtain maximum information from the animals we have employed in the study. Nonetheless, we have an ambitious schedule in Year 4 in order to keep this project on target.

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APPENDICES

TABLE 1: Hematological parameters of 1-month implantation groups

Group	WBC	RBC	HGB	HCT	PLT
	10^3/µl	10^6/µl	g/dL	%	10^3/µl
Sham	5.03 ± 0.35	8.48 ± 0.22	13.70 ± 0.23	41.75 ± 1.03	1117.00 ± 60.81
Ta	4.47 ± 0.46	8.37 ± 0.17	13.76 ± 0.36	40.76 ± 0.67	837.29 ± 159.39
Ni	4.29 ± 0.43	8.48 ± 0.11	12.86 ± 0.19 *	39.55 ± 0.46	1263.00 ± 59.55
Pb	5.24 ± 0.56	8.85 ± 0.08	13.96 ± 0.10	43.74 ± 0.50	1079.50 ± 53.37
WNiCo	5.39 ± 0.36	7.97 ± 0.24	12.70 ± 0.45	38.83 ± 1.06	950.75 ± 154.27
WNiFe	4.94 ± 0.37	8.75 ± 0.16	13.77 ± 0.22	42.68 ± 0.68	1218.33 ± 100.77
WTa	4.36 ± 0.45	8.99 ± 0.17	14.44 ± 0.22 *	44.58 ± 0.98	1289.88 ± 57.03
NiTa	6.39 ± 0.43 *	8.48 ± 0.19	13.89 ± 0.18	42.71 ± 0.95	1248.38 ± 93.94
CoTa	6.37 ± 0.66	8.67 ± 0.18	13.79 ± 0.27	42.06 ± 0.91	1201.38 ± 151.03
FeTa	7.14 ± 0.78 *	8.69 ± 0.18	13.80 ± 0.26	41.95 ± 0.57	1408.90 ± 60.52 *
WNiTa	6.58 ± 0.98	8.83 ± 0.13	13.79 ± 0.24	42.00 ± 0.58	1279.44 ± 86.05
WCoTa	5.93 ± 0.48	8.60 ± 0.15	13.63 ± 0.22	41.99 ± 0.74	1331.90 ± 71.51 *
WFeTa	6.61 ± 0.53 *	8.71 ± 0.19	14.14 ± 0.22	43.42 ± 0.88	1322.89 ± 50.30 *
NiFeTa	5.78 ± 0.35	8.85 ± 0.16	14.00 ± 0.23	44.04 ± 0.82	1296.20 ± 38.97 *
NiCoTa	5.87 ± 0.47	9.07 ± 0.12 *	14.22 ± 0.18	44.79 ± 0.63 *	1125.89 ± 31.66

Data represent the mean ± standard error. * denotes statistical significance at $P<0.05$ as determined by one-way ANOVA.

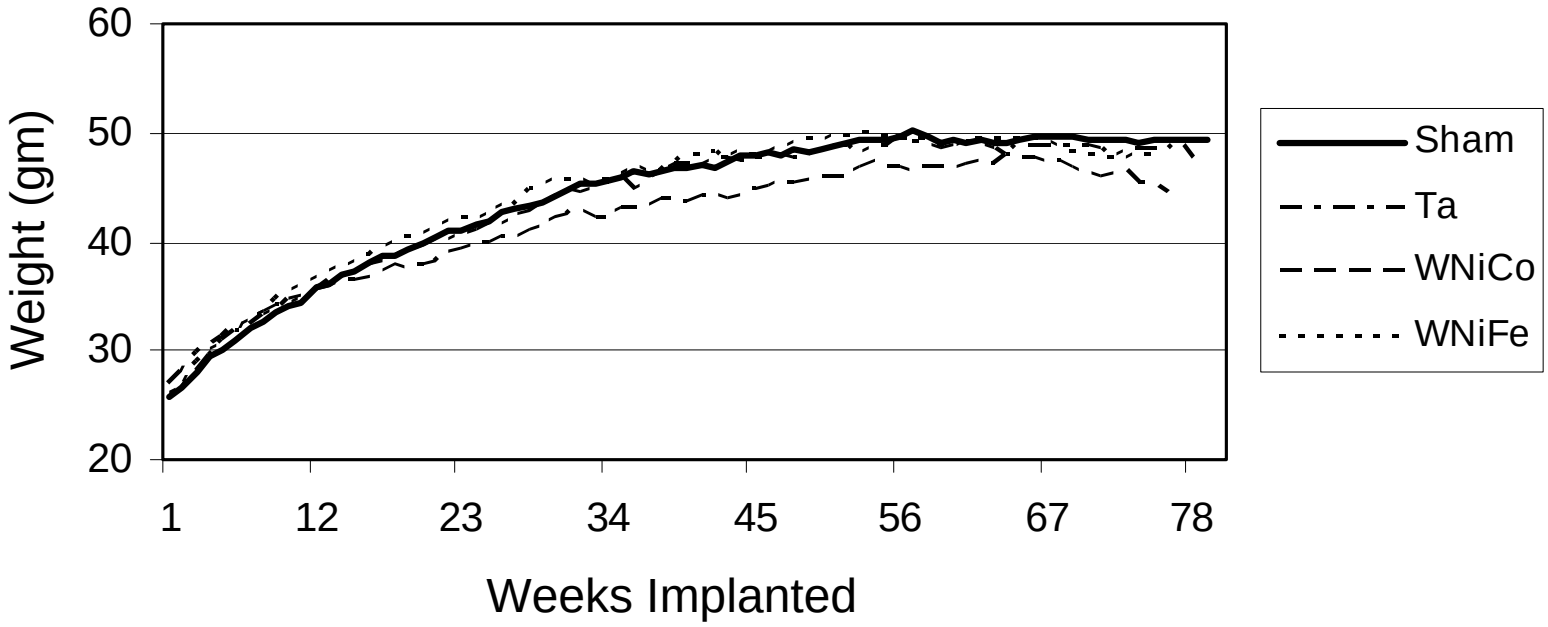

Fig. 1. Weight gain in high-dose 24 month groups (sham, Ta, WNiCo, WNiFe).

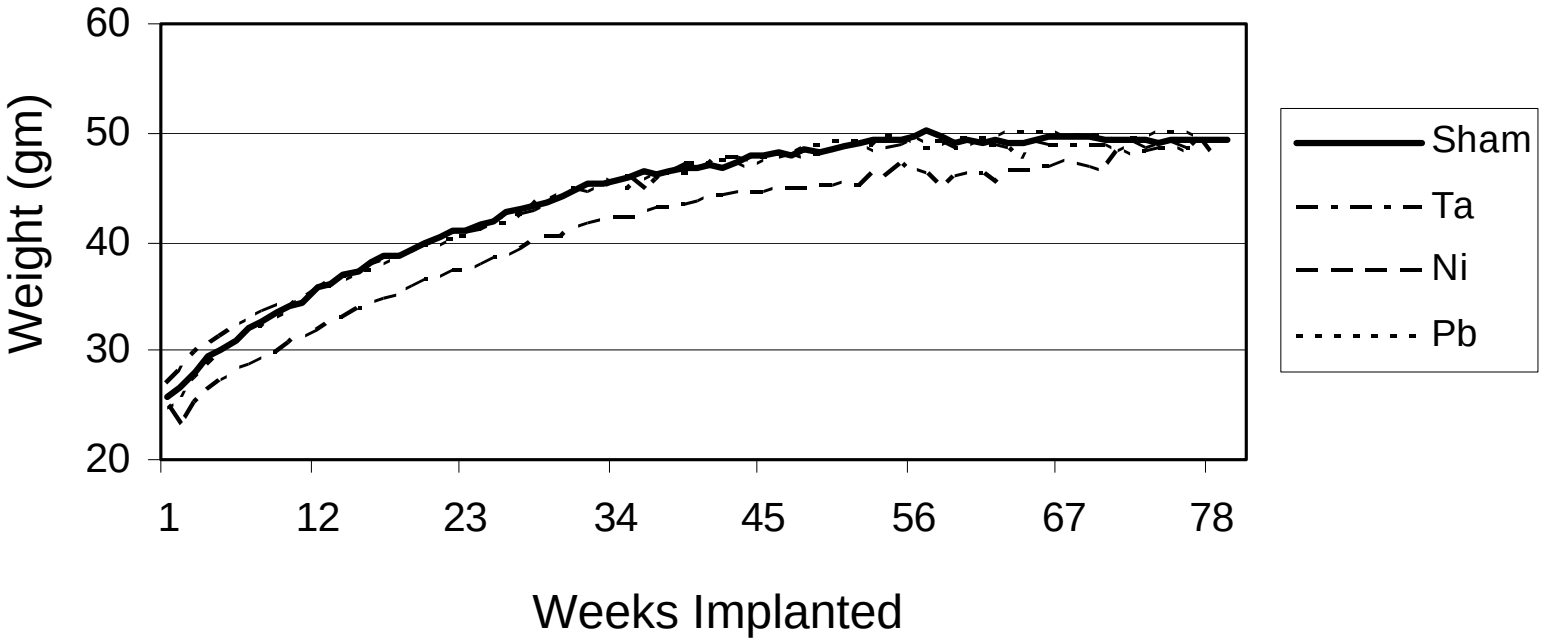

Fig. 2. Weight gain in high-dose 24 month groups (sham, Ta, Ni, Pb).

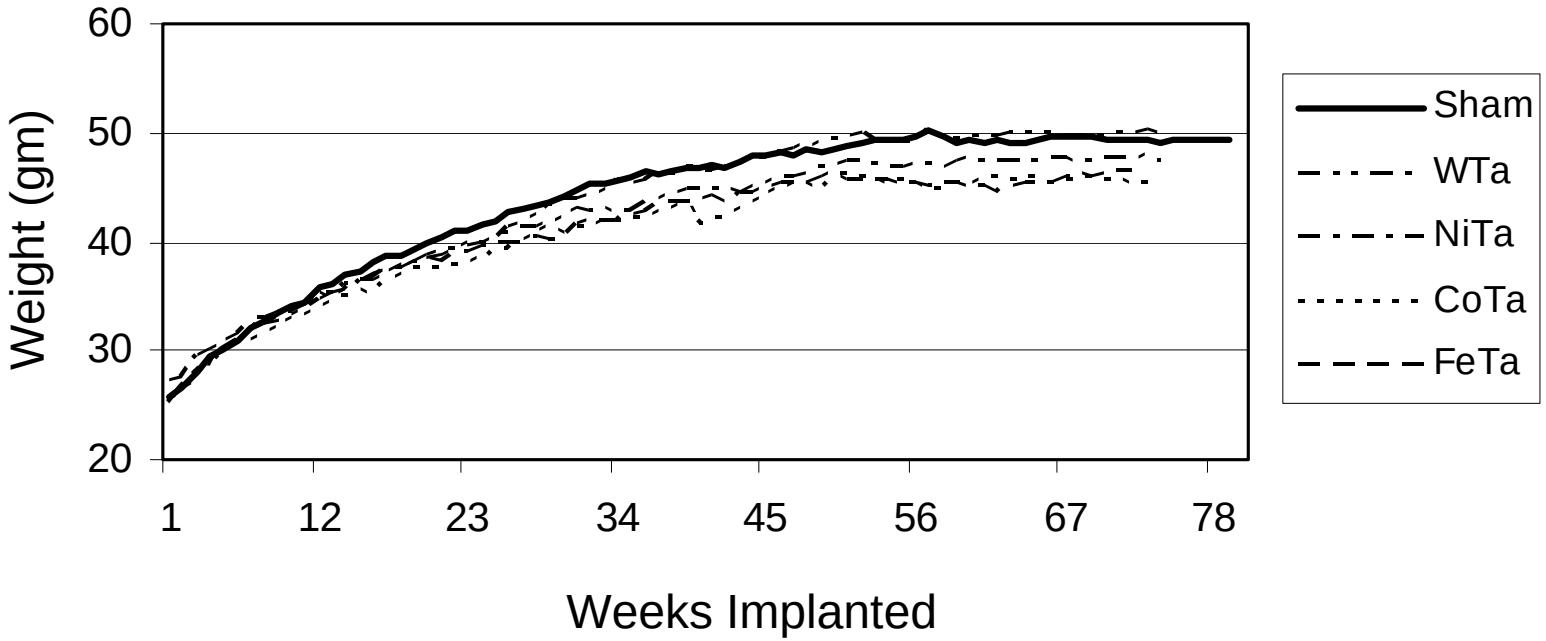

Fig. 3. Weight gain in high-dose 24 month groups (sham, WTa, NiTa, CoTa, FeTa).

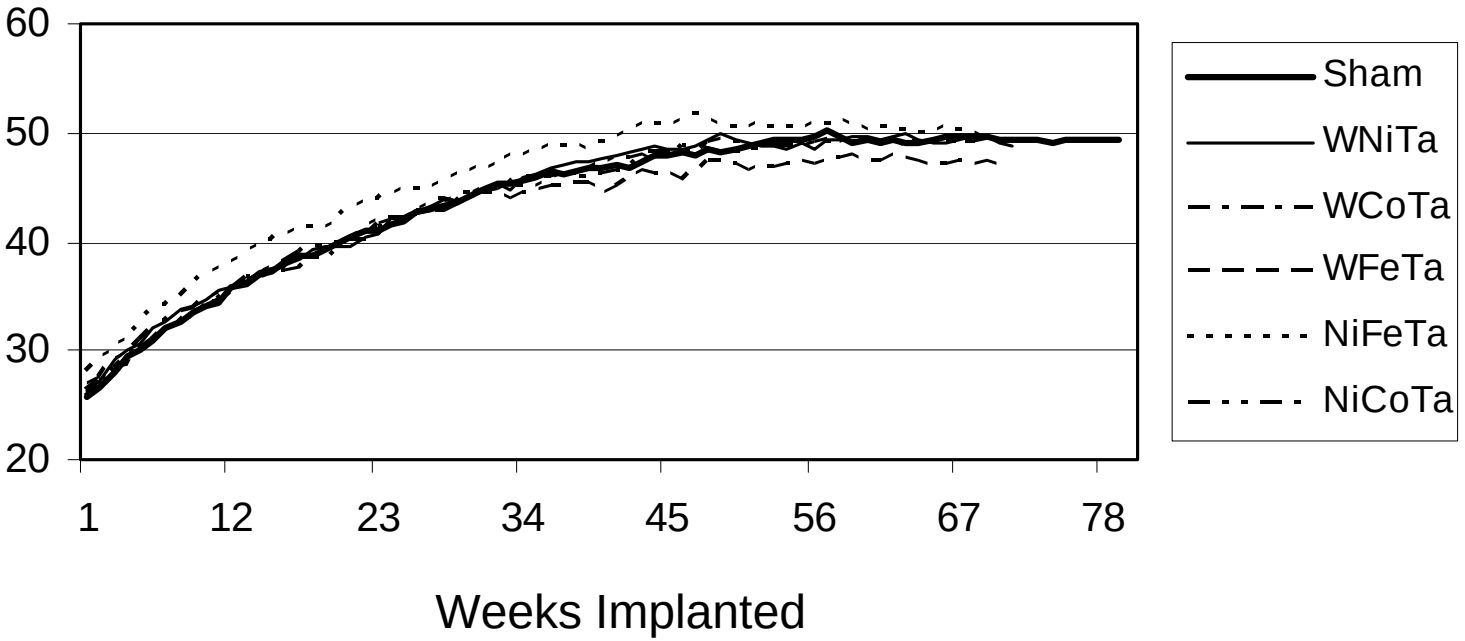

Fig. 4. Weight gain high-dose 24 month groups (sham, WNiTa, WCoTa, WFeTa, NiFeTa, NiCoTa).

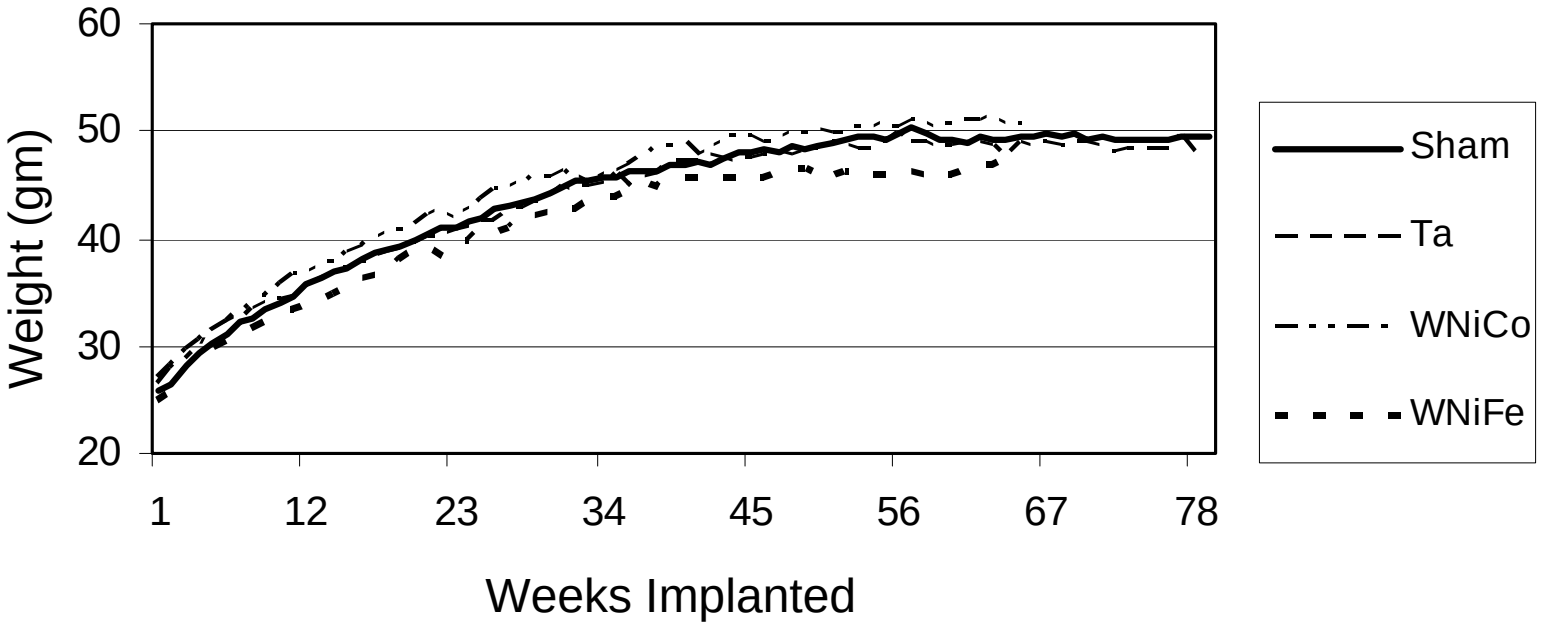

Fig. 5. Weight gain in low-dose 24 month groups (sham, Ta, WNiCo, WNiFe).

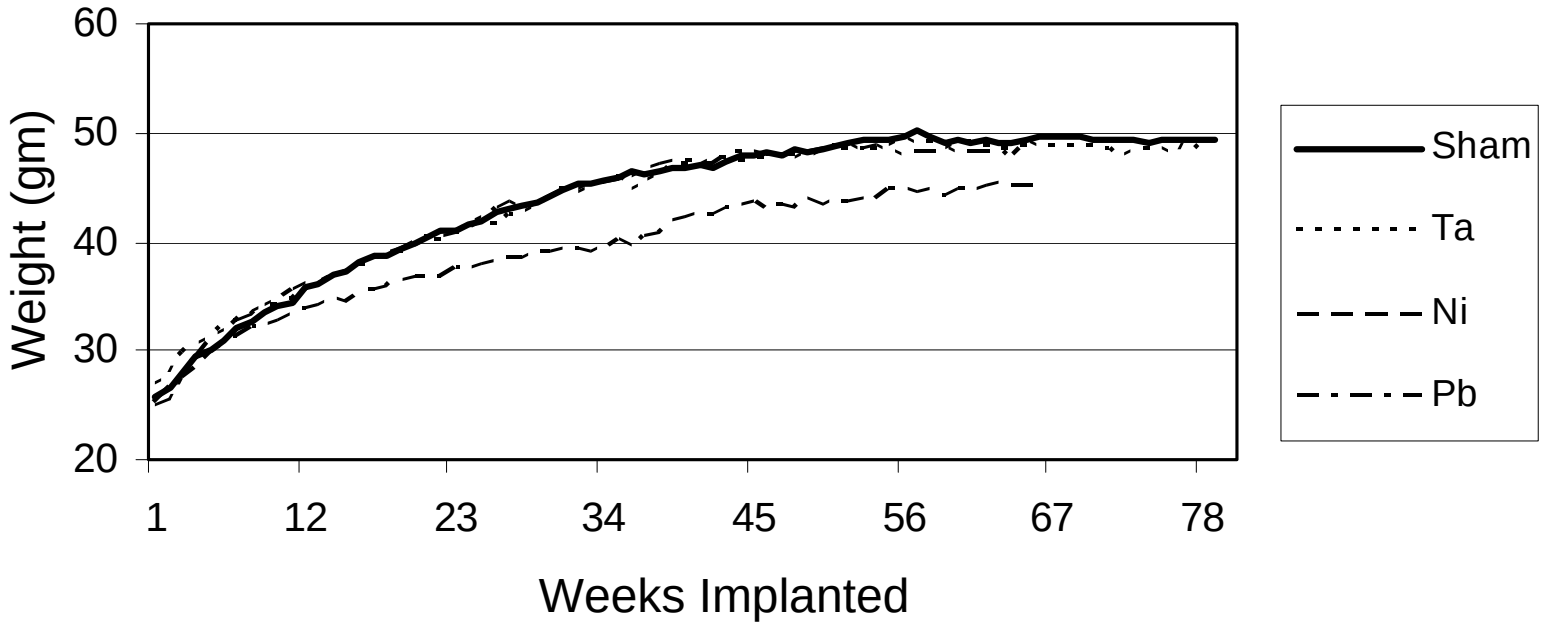

Fig. 6. Weight gain in low-dose 24 month groups (sham, Ta, Ni, Pb).

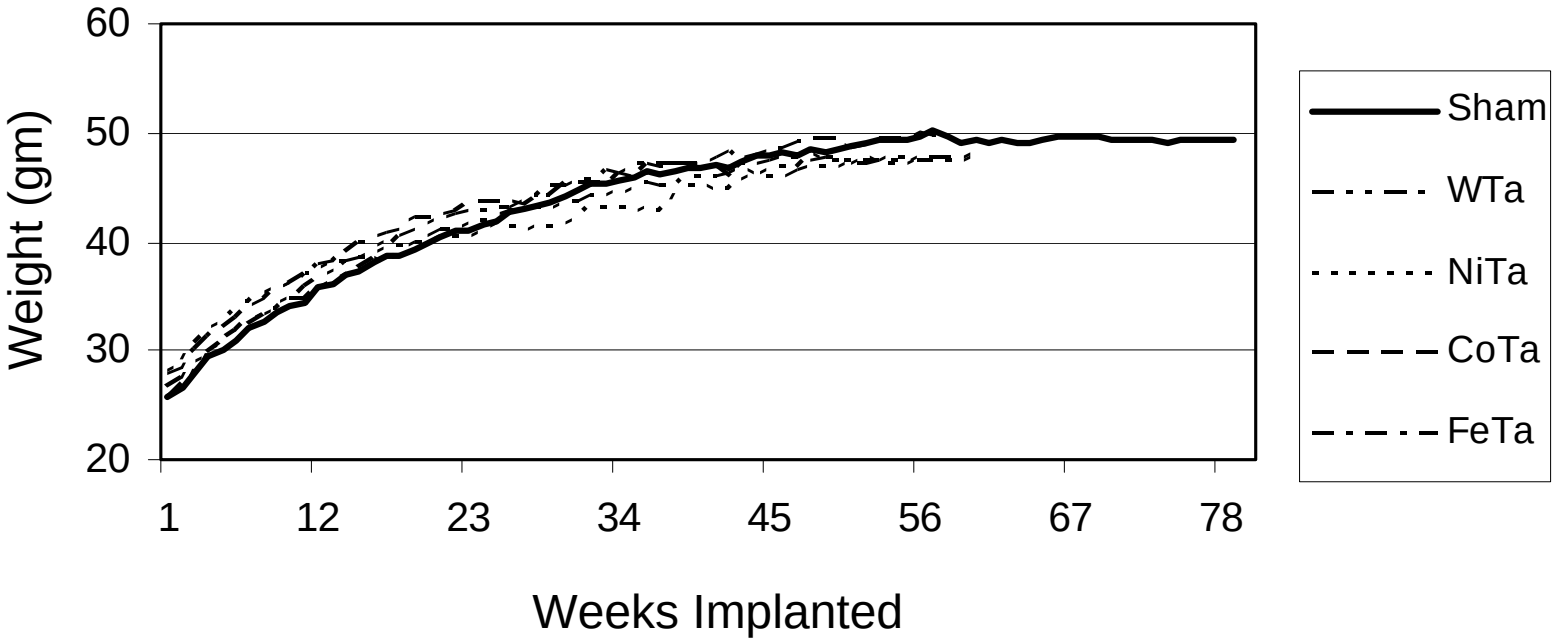

Fig. 7. Weight gain in low-dose 24 month groups (sham, WTa, NiTa, CoTa, FeTa).

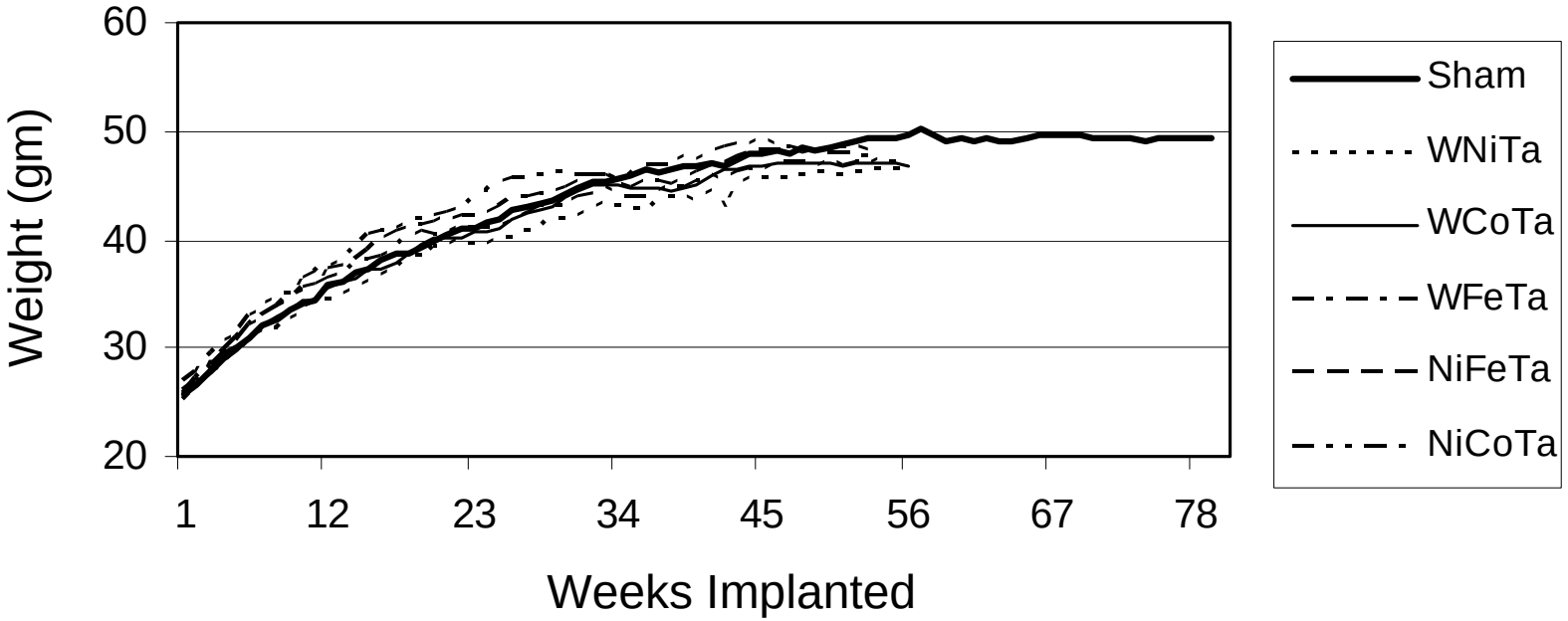

Fig. 8. Weight gain in low-dose 24 month groups (sham, WNiTa, WCoTa, WFeTa, NiFeTa, NiCoTa).

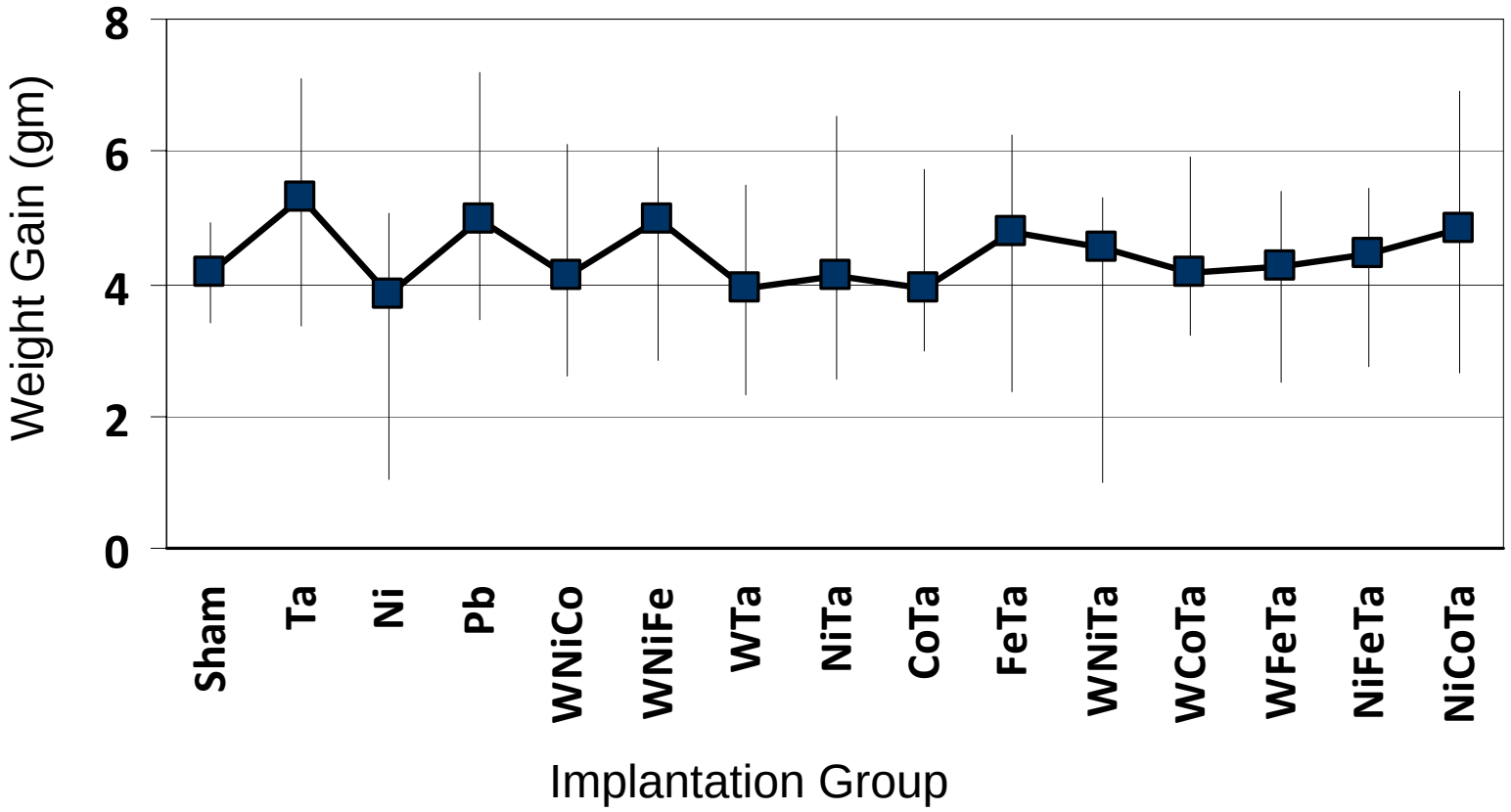

Fig. 9. Mean weight gain in 1 month implantation groups. Vertical lines indicate weight gain range.

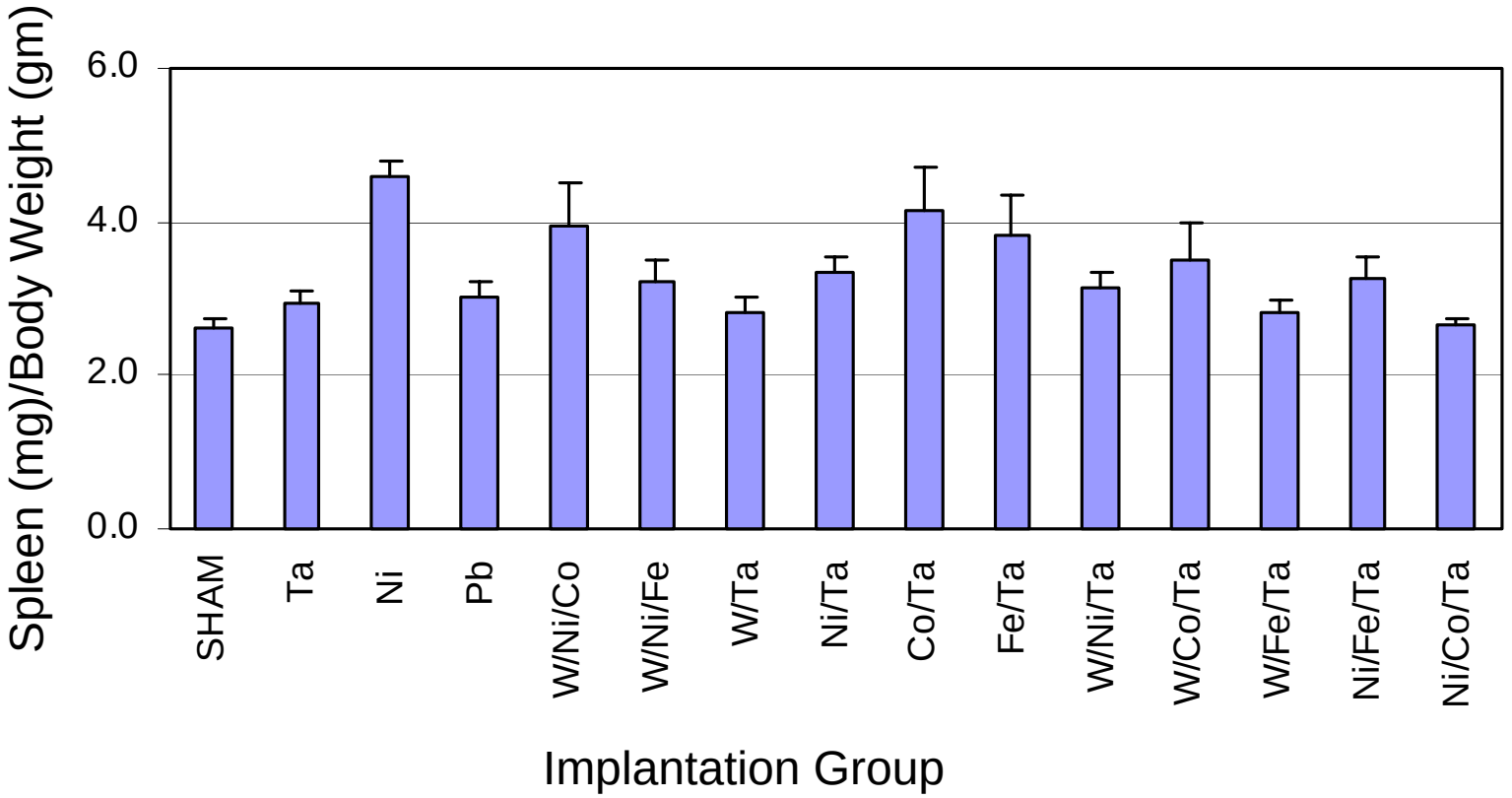

Fig. 10. Spleen/body weight ratios from 1 month implantation groups.

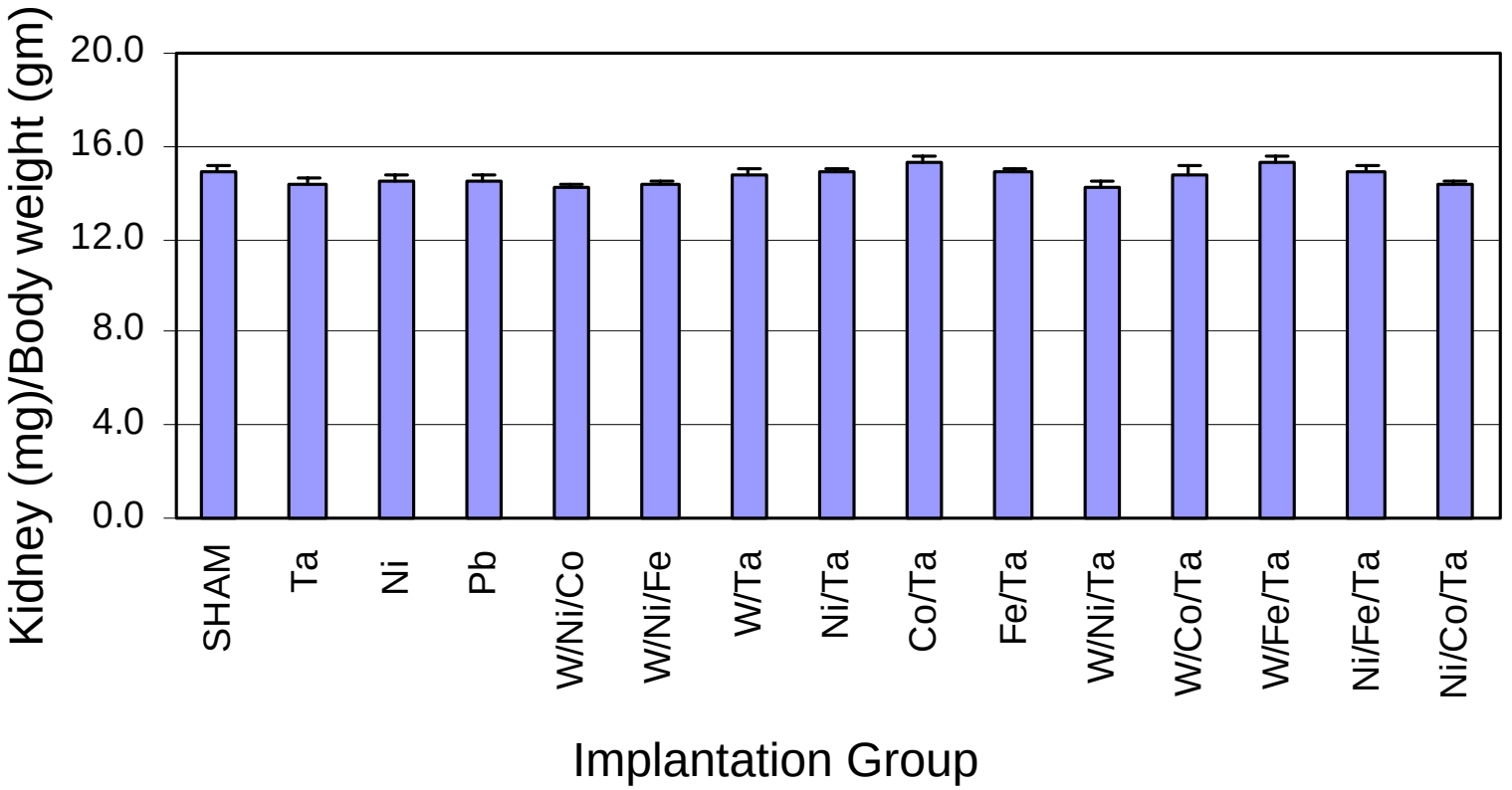

Fig. 11. Kidney/body weight ratios for 1 month implantation groups.

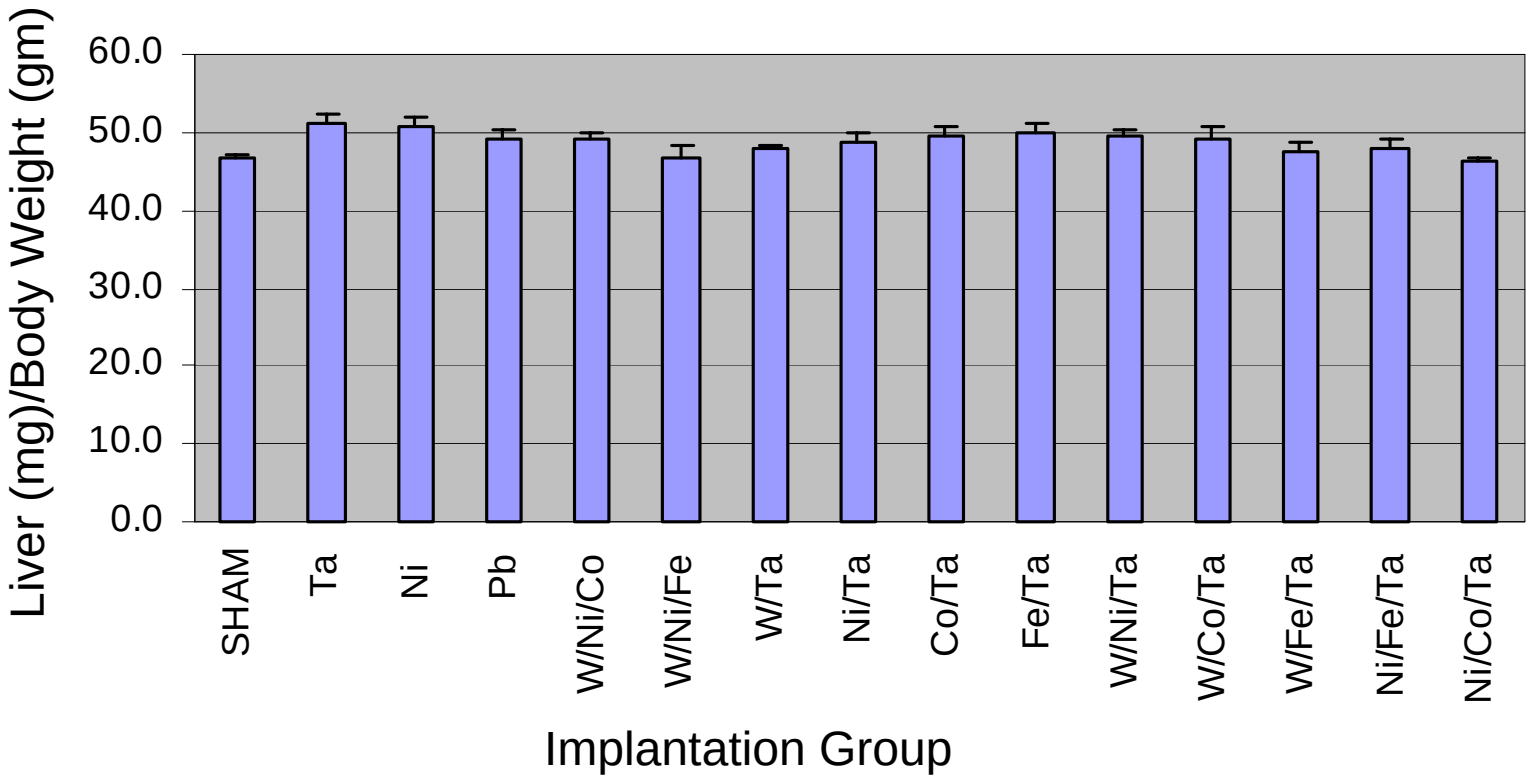

Fig. 12. Liver/body weight ratios for 1 month implantation groups.

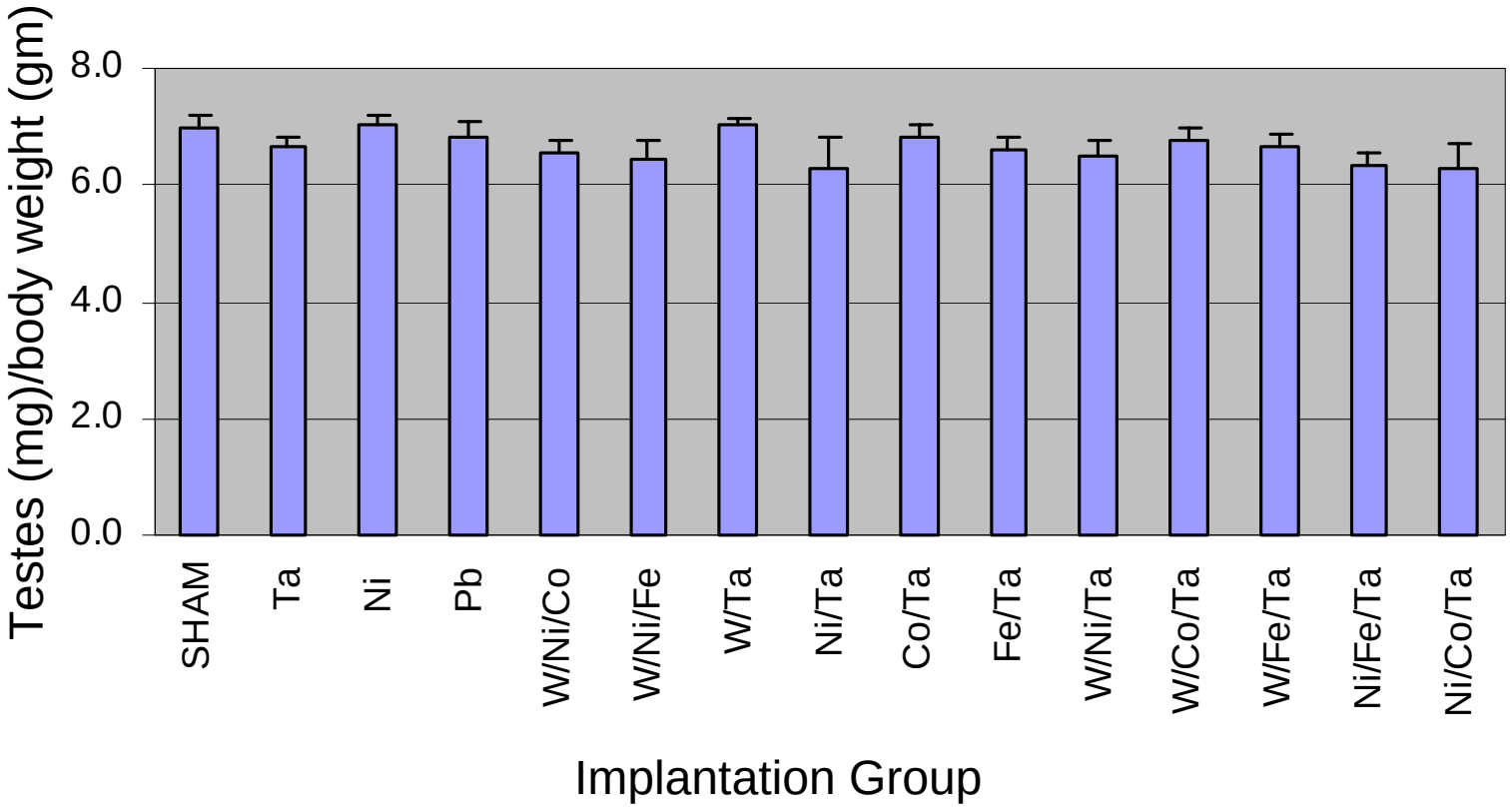

Fig. 13. Testes/body weight ratios in 1 month implanation groups.